

## **EXHIBIT 9**

## Development of Insulinitis without Diabetes in Transgenic Mice Lacking Perforin-dependent Cytotoxicity

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### Summary

It is widely accepted that T cells play an important role in the destruction of  $\beta$  cells leading to autoimmune type I diabetes, but the involved effector mechanisms have remained unclear. We addressed this issue by testing the role of perforin-dependent cytotoxicity in a disease model involving transgenic mice expressing glycoprotein of lymphocytic choriomeningitis virus (LCMV-GP) in the  $\beta$  cells of the endocrine pancreas. In such mice, LCMV infection leads to a potent LCMV-GP-specific T cell response resulting in rapid development of diabetes. We report here that in perforin-deficient LCMV-GP transgenic mice, LCMV infection failed to induce diabetes despite the activation of LCMV-GP-specific T cells. Deletion of  $\nu\beta 6^+$  T cells in Mls-1<sup>a</sup> perforin-deficient mice and the activation of LCMV-GP-specific T cells in perforin-deficient LCMV-GP transgenic mice, however, indicated that thymic tolerance induction by negative selection was not affected by the disruption of the perforin gene and that there is no fundamental difference between the T cell repertoires of normal control and perforin-deficient mice. In addition, adoptive transfer of LCMV-GP-specific TCR transgenic perforin-deficient T cells activated by LCMV-GP recombinant vaccinia virus led to marked insulinitis with infiltration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells without the development of diabetes. These findings indicate that perforin-dependent cytotoxicity is not required for the initiation of insulinitis but is crucial for the destruction of  $\beta$  cells in the later phase of the disease process. Other mechanisms or soluble factors present in the inflammatory islet infiltrate apparently lack the ability to efficiently induce diabetogenic  $\beta$  cell damage.

Insulin-dependent diabetes mellitus is a progressive autoimmune disease resulting in destruction of more than 90% of insulin-producing  $\beta$  cells in the pancreatic islets. The extensive study of spontaneous diabetes in the non-obese diabetic (NOD)<sup>1</sup> mouse has greatly promoted the understanding of the pathogenic mechanisms involved in the induction and progression of the disease. It is widely accepted now that T cells are crucially involved in the development of diabetes in NOD mice. This conclusion is based on the observation that a majority of islet-infiltrating cells are T cells (1), that diabetes can be transferred to young irradiated NOD mice with T cells from diabetic animals (2, 3), and that T cell depletion by specific antibodies is able to prevent diabetes (4). But the role of the CD4<sup>+</sup> helper and CD8<sup>+</sup> cytotoxic T cell subset in inducing and perpetuating the disease is controversial. Transfer of T cell

subpopulations from diabetic animals to young NOD mice has shown that both populations are required for the induction of diabetes (5–7). On the other hand, injection of a CD4<sup>+</sup> T cell clone or of activated TCR transgenic T<sub>H1</sub> cells accelerated diabetes in young NOD mice (8, 9). To accommodate these somewhat conflicting data two discrete diabetogenic effector mechanisms were proposed. The first mechanism is mediated by MHC class I-restricted CD8<sup>+</sup> T cells inducing destruction of  $\beta$  cells by contact-dependent cytotoxicity. The second is mediated by MHC class II-restricted CD4<sup>+</sup> T cells that recruit nonspecific effector cells into the islets by releasing chemotactic factors. The observation that cloned CD4<sup>+</sup> T cells were able to induce diabetes in the absence of CD8<sup>+</sup> T cells indicated that although CD8<sup>+</sup> T cells under normal conditions may be the more efficient effector cells of  $\beta$  cell destruction, CD4<sup>+</sup> T cells are able to mediate the disease, given enough time, numbers, and high specificity.

Additionally, several findings supported a role for CD8<sup>+</sup> T cells in the early induction phase of diabetes in NOD mice. First, CD8<sup>+</sup> T cells appear early in the islets of predi-

<sup>1</sup>Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; LCMV-GP, glycoprotein of LCMV; vacc-LCMV-GP, vaccinia virus recombinant for LCMV-GP; NOD, non-obese diabetic.

abetic mice before CD4<sup>+</sup> T cells, which are detected only later in disease progression (10). Second,  $\beta_2$ -microglobulin-deficient NOD mice with an almost complete lack of CD8<sup>+</sup> T cells do not only fail to develop diabetes but also lack lymphocytic infiltration into the islets (11–13). These results suggested a model in which early infiltrating CD8<sup>+</sup> T cells cause a low level of islet cell lysis. The resulting release of  $\beta$  cell proteins could prime and/or augment a CD4<sup>+</sup> T cell response, which in turn is maintaining an inflammatory process involving the recruitment of other effector cells with toxicity for  $\beta$  cells.

So far, these experiments were not able to distinguish between effects of T cells mediated by cytokines, on the one hand, and contact-dependent cytotoxicity, on the other hand. Any effector mechanism has to account for the extreme specificity of the disease process that leads to elimination of islet  $\beta$  cells but spares  $\alpha$  cells and other minor islet cell types. A recent hypothesis proposes that IL-1 produced by cells in the inflammatory islet foci may account for specific  $\beta$  cell damage by selectively inducing the formation of nitric oxide in  $\beta$  but not in  $\alpha$  cells (14, 15).

To investigate the role of T cell-mediated contact-dependent cytotoxicity in autoimmune diabetes, we used perforin-deficient mice. Perforin, which is expressed in cytoplasmic granules of CTL and NK cells, is crucially involved in T and NK cell-mediated cytotoxicity and in effector functions of T cells and NK cells *in vivo* (16–21). Since perforin-deficient mice have no defect in ontogeny, activation, and proliferation of T cells (16), they offer a direct approach to address the role of effector mechanisms in the destruction of  $\beta$  cells in diabetes.

Perforin-deficient mice were bred with transgenic mice expressing the glycoprotein of lymphocytic choriomeningitis virus (LCMV-GP) (22, 23) in  $\beta$  cells of the pancreas. Normally, these mice are unresponsive but not tolerant to LCMV-GP. However, upon activation of LCMV-GP specific T cells by infection with LCMV they develop insulinitis and diabetes in 9 to 11 d, which is dependent on CD8<sup>+</sup> T cells. This study documents that in this model system of autoimmune diabetes the disease process is dependent upon perforin-dependent cytotoxicity.

## Materials and Methods

**Mice.** Perforin-deficient (16), LCMV-GP-specific T cell receptor transgenic and normal control C57BL/6 mice were bred under specific pathogen-free conditions at the Institut für Labor-tierkunde (University of Zürich, Switzerland). Since embryonic stem cells of C57BL/6 origin were used to generate these mice, they represent a mutant of the C57BL/6 strain. TCR transgenic mice were backcrossed in C57BL/6 background. All experiments were carried out with age (8–16 wk) and sex-matched animals.

**Breeding of TCR-transgenic Perforin-deficient Mice.** The well-characterized line 318 (24, 25) of transgenic mice expressing a T cell receptor specific for the LCMV-GP nonapeptide GP33 (amino acids 33–41) in the context of the D<sup>b</sup> molecule was bred with perforin-deficient mice. The resulting offspring were screened by cytometry for expression of the transgenic  $v_{\alpha 2}$  segment. Trans-

genic mice, which were heterozygous at the perforin locus, were then bred *inter se* to generate homozygous perforin-deficient TCR transgenic mice. The perforin genotype was determined with PCR and two different primer pairs on DNA prepared from tail biopsies. The first pair (5'-TTT TTT GGA CCC TGT AGA CCC A-3', 5'-GCA TCG CCT TCT ATC GCG TTC T-3') yields a band of 665 bp for the mutated and is negative for the wild-type allele. The second pair (5'-CGG GTC CTG AAC TCG TGG CCA A-3', 5'-CCC CTG CAC ACA TTA CTG GAA G-3') yields a 300-bp fragment for the wild-type and a 1,300-bp fragment for the mutated allele.

**Breeding of LCMV-GP Transgenic Perforin-Deficient Mice.** Mice expressing a LCMV-GP transgene under the control of the rat insulin promoter have been previously described (22, 23). The C57BL/6 line Bln of these transgenic mice was crossed with perforin-deficient mice and the offspring were subsequently bred *inter se*. Screening for the perforin locus was as above. The LCMV-GP transgene was detected by PCR (primers: 5'-CAA GCA AGA TGT AGA GTC TGC C-3', 5'-GGC TTT GGA CAT GAA CCG CCC-3') resulting in a 450-bp fragment.

**Cytofluorometry.**  $v_{\alpha 2}$  expressing T cells were measured in blood by incubating blood cells at 4°C in BSS containing 2% FCS and 0.2% NaN<sub>3</sub> with rat anti-mouse  $v_{\alpha 2}$  monoclonal antibody 44-22-1 followed by FITC-conjugated goat anti-rat IgG (Southern Biotechnology, Birmingham AL). After washing and saturation of residual second stage reagent with rat serum, the cells were stained with PE-conjugated CD4-specific antibody (Becton Dickinson, Mountain View, CA). Erythrocytes were lysed with FACS<sup>®</sup> lysis solution (Becton Dickinson) and the cell suspensions were analyzed on a FACScan<sup>®</sup> flow cytometer (Becton Dickinson) using logarithmic scales. Viable lymphocytes were gated for by a combination of forward light scatter and 90° side scatter. T cells expressing  $v_{\alpha 2}$  are shown as histograms gated on CD4<sup>+</sup> T cells.

To detect transgenic TCR expression, peripheral blood cells were stained with PE-conjugated  $v_{\alpha 2}$ -specific mAb B20.1 (Pharmingen, San Diego, CA) (26) and FITC-conjugated CD8-specific antibody (Becton Dickinson). After lysis of erythrocytes with FACS lysis solution (Becton Dickinson) and washing, cell suspensions were analyzed on a FACScan flow cytometer (Becton Dickinson) using logarithmic scales. Viable cells were gated for by a combination of forward light scatter and 90° side scatter.

**Cytotoxicity Assay.** RMA and MC57G target cells (both H-2<sup>b</sup>) were grown *in vitro* by standard cell culture techniques. They were labeled either with or without  $10^{-10}$  M LCMV-GP-derived epitope peptide GP33 (amino acids 33–41, KAVYNFATM (27)) with Na<sub>2</sub><sup>35</sup>CrO<sub>4</sub> (Amersham Int., Bucks., UK) on a rocking platform for 2 h at 37°C. Target cells were washed and used at  $10^4$  cells per well in round-bottomed 96-well plates mixed at various ratios with spleen effector lymphocytes in a final volume of 200  $\mu$ l. The effector and target cells were pelleted by centrifugation and incubated for 5 h at 37°C in the presence of 5% CO<sub>2</sub>. Total release was determined by incubating target cells with 0.5 M HCl. 70  $\mu$ l supernatant was removed and counted in an automated counting station (Cobra; Canberra-Packard, Meriden, CT), which calculated percent specific release as (sample release – spontaneous release) / (total release – spontaneous release)  $\times 100$ .

**Viruses.** LCMV-WE was originally obtained from Dr. F. Lehmann-Grube (Hamburg, Germany) and propagated on L929 cells. Recombinant vaccinia virus expressing the full length of LCMV glycoprotein precursor molecule (vacc-LCMV-GP) was a gift from Dr. D.H.L. Bishop (Institute of Virology, Oxford University, Oxford UK) (28, 29). Recombinant vaccinia virus stocks were grown on BSC 40 cells. The cells and culture supernatants

were collected 2 d after infection, subjected to three cycles of freezing and thawing, sonicated on ice for 30 s and centrifuged.

**Measurement of Blood Glucose.** The glucose concentration in blood obtained from a tail vein was measured using Haemo-Glucotest strips (Böhringer, Mannheim, Germany).

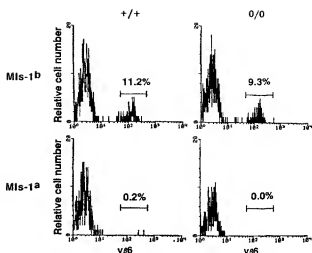
**Immunohistochemistry.** Pancreata were immersed in Hank's balanced salt solution and snap frozen in liquid nitrogen. Cryostat sections (5  $\mu$ m) of tissue were cut and fixed in cold acetone. Sections were incubated with rat anti-mouse monoclonal antibodies YTS191.1 (anti CD4), YTS169.4.2 (anti-CD8) (30) or F4/80 (anti-macrophage) (31), respectively. Alkaline phosphatase-labeled goat anti-rat antibodies, followed by alkaline phosphatase-labeled donkey anti-goat antibodies (Tago, Burlingame, CA) were used as secondary reagents. The substrate for the red color reaction was naphthol AS-BI phosphate/New Fuchsin. Insulin was detected on paraffin-embedded formalin fixed tissues with the insulin-specific monoclonal antibody AE9D6 (BioGenex Laboratories, San Ramon, CA). Primary antibodies were detected by an indirect immunoenzymatic staining procedure with biotinylated anti-immunoglobulin antibodies and a biotin/streptavidin-amplified horseradish peroxidase detection system (BioGenex Laboratories). The brown color reaction was developed with diaminobenzidine.

**Adoptive Transfer.** Perforin-competent or perforin-deficient mice, both transgenic for a LCMV-GP-specific TCR, were i.v. infected with  $2 \times 10^6$  PFU vacc-LCMV-GP to activate the LCMV-GP-reactive transgenic T cells. After 6 d,  $5 \times 10^6$  spleen cells were adoptively transferred into perforin-deficient mice expressing LCMV-GP in the pancreas. To keep the adoptively transferred T cells in an activated state, the recipients were i.v. infected with  $2 \times 10^6$  pfu vacc-LCMV-GP. Diabetes was checked by measuring blood glucose levels.

## Results

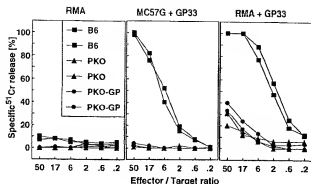
**Negative Selection and Deletion of TCR- $v_{\beta 6}^+$  T Cells Is Not Influenced by the Absence of Perforin.** Autoimmune diseases including diabetes type 1 may ensue due to failure of tolerance establishment to self antigens, which subsequently become the target of an inflammatory disease process. To assess whether the lack of perforin affects tolerance induction by deletion of autoreactive cells (32), perforin-deficient Mls-1<sup>b</sup> mice were generated by crossing with mice of the Mls-1<sup>a</sup>-expressing DBA/2 strain. The resulting F1 mice were then bred inter se to achieve homozygosity for the disrupted perforin allele. The presence of Mls-1<sup>a</sup> was tested by a PCR reaction detecting the endogenous provirus form of the mouse mammary tumour virus MMTV(SW) (33). Cytofluorometric analysis of spleen cells from perforin-expressing Mls-1<sup>b</sup>, perforin-expressing Mls-1<sup>a</sup>, perforin-deficient Mls-1<sup>b</sup> and perforin-deficient Mls-1<sup>a</sup> mice showed that Mls-1<sup>a</sup>-specific  $v_{\beta 6}^+$  T cells were absent in perforin-deficient as well as in perforin-expressing Mls-1<sup>a</sup> mice. Thus, negative selection is independent of the cytotoxic protein perforin and occurs normally in perforin-deficient mice.

**LCMV-GP-specific Cytotoxic T Cell Responses of LCMV-GP Transgenic Mice.** LCMV-GP transgenic mice have been studied previously (22). It has been found that potentially autoreactive LCMV-GP-reactive cells in these mice are present without undergoing negative selection or energy.



**Figure 1.** Deletion of Mls-1 reactive TCR- $v_{\beta 6}$  expressing T cells in the absence of perforin.  $v_{\beta 6}$  expression in peripheral blood is shown as histograms of CD4<sup>+</sup> T cells. Mls-1<sup>b</sup> perforin-expressing C57BL/6, Mls-1<sup>a</sup> perforin-expressing DBA/2, Mls-1<sup>b</sup> perforin-deficient mice (C57BL/6 genetic background) and Mls-1<sup>a</sup> perforin-deficient mice were tested by cytofluorometry for the presence of Mls-1 reactive TCR- $v_{\beta 6}$  expressing T cells. The latter mouse strain was generated by outcrossing perforin-deficient C57BL/6 mice to the Mls-1<sup>a</sup>-expressing strain DBA/2.

Instead, these T cells ignore antigen expressed in the islets of the pancreas and remain quiescent unless they are activated by an infection with LCMV. This leads to a proliferative and cytotoxic LCMV-GP-specific T cell response comparable to nontransgenic mice. The T cell response also induces infiltration of LCMV-GP-specific T cells into the pancreas and diabetes. Thus, a prerequisite to test the role of perforin-dependent cytotoxicity with perforin-deficient LCMV-GP transgenic mice is that activation of LCMV-GP-specific T cells is not impaired by partial or complete tolerance mechanisms. To formally prove this point, C57BL/6 normal control mice, perforin-deficient nontransgenic and perforin-deficient LCMV-GP transgenic mice were infected with 200 PFU LCMV-WE i.v. On day 8 after infection, spleen cells were tested for cytotoxic activity on various target cells. We have shown previously (16, 34, 35) that MC57G cells due to their lack of Fas-expression are susceptible only to perforin-dependent cytotoxicity, whereas Fas-expressing RMA target cells are susceptible to perforin- and Fas-dependent cytotoxicity. Thus, cytotoxic activity of perforin-deficient effector cells from LCMV-GP transgenic and nontransgenic mice on MC57G target cells labeled with the LCMV-GP epitope nonapeptide GP33 (LCMV-GP aminoacids 33–41) (27) was completely absent (Fig. 2). On RMA cells incubated with GP33 peptide, in contrast, the cytotoxic activity of perforin-deficient effector cells, which is mediated by the interaction of Fas-ligand on the T cell with Fas expressed on the surface of the target cell (19, 34, 36–38), was markedly reduced compared to the activity of C57BL/6 effector cells but clearly detectable. Spleen cells from LCMV-GP

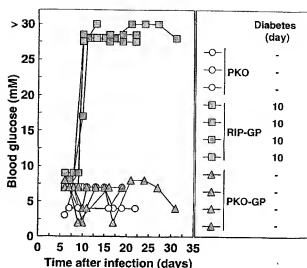


**Figure 2.** Ability of LCMV-GP transgenic perforin-deficient mice to generate a LCMV-GP specific Fas-dependent cytotoxic T cell response. Normal control C57BL/6 mice (B6), perforin-deficient (PKO), and LCMV-GP transgenic perforin-deficient (PKO-GP) mice were infected with 200 PFU LCMV-WE. After 8 d, the cytotoxic activity of spleen cells was tested on Fas-negative MC57G cells and Fas-expressing RMA cells. The target cells were either labeled with a main epitope peptide of LCMV-GP (GP33) or used without peptide to demonstrate specificity. The experiment was repeated twice with similar result.

transgenic and nontransgenic perforin-deficient mice specifically lysed labeled RMA cells with similar efficiency indicating that the LCMV-GP transgene did not induce negative selection or anergy of potentially autoreactive cells and that LCMV-GP-reactive T cells are present in the same numbers in transgenic and nontransgenic perforin-deficient mice.

**Diabetes Induction by LCMV Infection in Perforin-expressing but Not in Perforin-deficient LCMV-GP Transgenic Mice.** Perforin-deficient LCMV-GP transgenic mice are healthy and do not develop spontaneous insulinitis. As previously reported (22), perforin-competent LCMV-GP transgenic mice develop insulinitis and diabetes between day 9 and 11 after LCMV infection. To test the role of perforin-dependent cytotoxicity in the induction of diabetes, perforin-competent and perforin-deficient LCMV-GP transgenic mice were infected with 200 PFU LCMV-WE i.v.. The onset of diabetes was determined by measuring the level of blood glucose in regular intervals. In contrast to perforin-competent mice, perforin-deficient LCMV-GP transgenic mice did not develop diabetes (Fig. 3). Perforin-deficient mice do, however, develop weight loss of up to 20% after infection and eventually die between day 15 and 25 as described earlier (16). However, this pathology was significantly slower than the induction of diabetes in perforin-expressing mice allowing the observation of perforin-deficient mice for several days beyond the onset of diabetes in LCMV-GP transgenic perforin-expressing control mice. We found that whereas all of the perforin-competent mice developed diabetes on day 10 after LCMV infection, the perforin-deficient LCMV-GP transgenic mice failed to develop hyperglycemia. One of four perforin-deficient LCMV-GP transgenic mice survived infection even up to 32 d without developing diabetes.

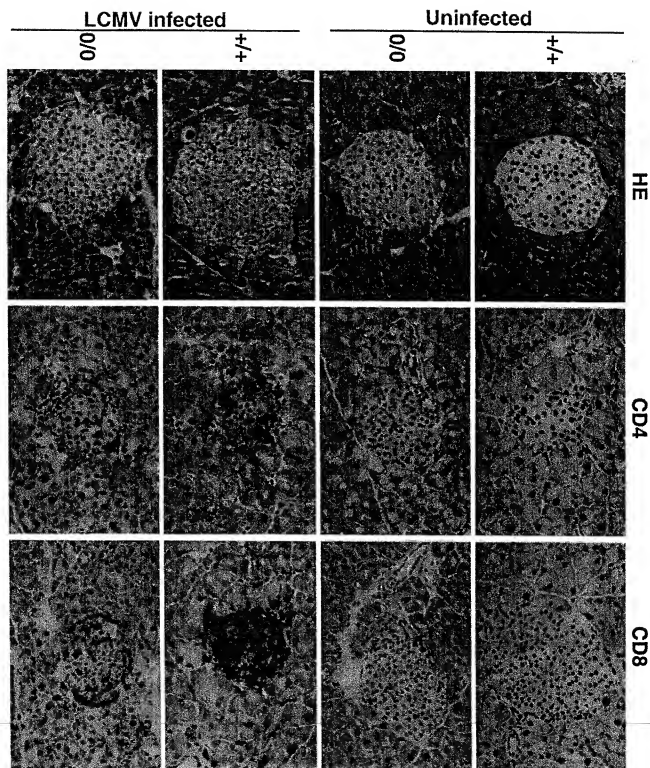
**Histological Analysis of Insulinitis in Perforin-Deficient LCMV-GP Transgenic Mice.** Pancreas pathology was further characterized



**Figure 3.** Failure of LCMV-GP transgenic perforin-deficient mice to develop diabetes upon LCMV infection. Perforin-deficient (PKO), LCMV-GP transgenic (RIP-GP) and perforin-deficient LCMV-GP transgenic (PKO-GP) mice were infected with 200 PFU LCMV i.v. Diabetes was detected by monitoring blood glucose levels after infection. Perforin-deficient LCMV-GP transgenic as well as perforin-deficient nontransgenic mice suffered from marked weight loss upon LCMV infection and died without developing diabetes.

by histological analysis on day 9 (Fig. 4). This time point, shortly before diabetes onset in perforin-competent LCMV-GP transgenic mice, was chosen because it should be most informative about the lymphocyte populations involved in the depletion of pancreatic  $\beta$  cells. Both, perforin-competent and perforin-deficient LCMV-GP mice were free of insulinitis before infection (Fig. 4, upper two rows). Upon LCMV infection, marked lymphocytic infiltration of the pancreas in perforin-expressing LCMV-GP transgenic mice developed and was accompanied by a loss of secretory cells rich in cytoplasm. As previously described (22), CD4<sup>+</sup> T cells tended to be localized more on the periphery of islets whereas CD8<sup>+</sup> T cells infiltrated the entire islets. In perforin-deficient LCMV-GP transgenic mice, CD8<sup>+</sup> T cells infiltrating into the entire islets were detected, but their number was lower than in perforin-expressing LCMV-GP transgenic mice. Only few CD4<sup>+</sup> T cells were found in the pancreas of perforin-deficient mice and in most islets a considerable number of secretory cells rich in cytoplasm were present. Few F4/80 positive macrophages were detected in islets of normal control as well as of perforin-deficient mice (data not shown).

**Adoptive Transfer of Activated LCMV-GP-Specific T Cells.** LCMV-GP transgenic perforin-deficient mice fail to develop diabetes upon LCMV infection. This may be explained by a crucial role of perforin-dependent cytotoxicity exerted by CD8<sup>+</sup> T cells in the destruction of  $\beta$  cells in this model of autoimmune diabetes. However, since perforin-deficient mice are unable to control the growth of LCMV, which is eliminated in wild-type C57BL/6 mice by day 8

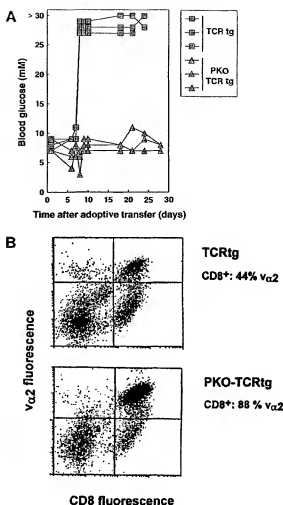


**Figure 4.** Histological analysis of pancreatic islets from LCMV-GP transgenic (+/+) perforin-expressing and perforin-deficient mice. Pancreatic sections from either uninfected or LCMV infected (day 9 after infection with 200 PFU LCMV-WE i.v.) were stained with standard HE stain or used for immunohistochemistry with CD4- or CD8-specific antibodies. The respective CD4- or CD8-expressing cells appear black.  $\times 400$ .

after infection, diversion of LCMV-GP-specific T cells by abundant virus-infected cells in perforin-deficient mice could account for the failure of perforin-deficient LCMV-GP transgenic mice to develop diabetes. In fact, the reduced lymphocytic infiltration observed in these mice would support this notion.

To exclude this latter possibility we avoided LCMV infection by using an experimental approach in which LCMV-GP-specific T cells are activated by infection with a LCMV-GP recombinant vaccinia virus strain (vacc-LCMV-GP). The main advantage of this experiment was that the virus load in normal control and perforin-deficient mice did not vary significantly because perforin-deficient mice have an intact ability to control a number of cytopathic viruses including vaccinia virus (39). Splenic T cells from vacc-GP infected mice were adoptively transferred to LCMV-GP transgenic perforin-deficient recipients. To achieve a higher frequency of LCMV-GP-reactive T cells, LCMV-GP-specific TCR transgenic perforin-expressing or perforin-deficient mice were used as donors. Whereas all of the recipients that had received perforin-expressing TCR transgenic spleen cells developed high glucose levels on day 8 after infection, recipients of perforin-deficient TCR transgenic cells remained normoglycemic during the whole observation period of 60 d (Fig. 5A). To exclude the possibility that spleen cells from perforin-deficient but not from perforin-expressing TCR-transgenic donors were rejected by the recipients due to some minor genetic differences, some recipients were infected with LCMV on day 15 after adoptive transfer. LCMV infection induces a much more vigorous proliferative response of LCMV specific CD8<sup>+</sup> T cells than vacc-LCMV-GP and therefore allowed the amplification of LCMV-specific transgenic T cells to easily detectable levels. 9 d after LCMV infection, the frequency of transgenic T cells was determined by cytometry with a TCR- $v_{\alpha 2}$ -specific antibody. The transgenic TCR- $v_{\alpha 2}$  segment, which is present in nontransgenic mice in less than 5% of CD8<sup>+</sup> T cells, was expressed on 88% of CD8<sup>+</sup> peripheral blood T cells in recipients of perforin-deficient vs. 44% in recipients of perforin-competent TCR transgenic spleen cells indicated that the failure of perforin-deficient spleen cells to induce diabetes upon adoptive transfer was not due to rejection of transferred cells (Fig. 5B). The more pronounced expansion of TCR-transgenic T cells in recipients of perforin-deficient spleen cells is probably explained by the failure of these animals to control LCMV infection, which in turn leads to stronger stimulation of LCMV-GP-specific T cells.

**Insulinitis and Diabetes after Adoptive Transfer of TCR Transgenic Cells.** Pancreatic sections of recipient mice were stained with HE-, CD4-, or CD8-specific antibodies. Immunohistological analysis of pancreatic sections was performed 10 d following adoptive transfer shortly after the recipients of perforin-expressing control spleen cells turned diabetic. As expected from the high glucose levels, recipients of perforin-competent spleen cells displayed extensive damage to the islets (Fig. 6, top). In fact, intact islets with secretory cells rich in cytoplasm were absent and only few infiltra-



**Figure 5.** Activated perforin-deficient T cells fail to induce diabetes upon adoptive transfer. (A) Perforin-competent (TCR tg) or perforin-deficient (PKO-TCRtg) mice transgenic for a LCMV-GP-specific TCR were infected i.v. with  $2 \times 10^6$  PFU vacc-LCMV-GP to activate the LCMV-GP reactive transgenic T cells. After 6 d,  $5 \times 10^6$  spleen cells were adoptively transferred into mice expressing LCMV-GP in the pancreas. To keep the adoptively transferred T cells in an activated state, the recipients were i.v. infected with  $2 \times 10^6$  PFU vacc-LCMV-GP. Diabetes was monitored by measuring blood glucose levels. The experiment was repeated twice with similar result. (B) Presence of TCR transgenic T cells in peripheral blood of recipients that were prepared as above but infected on day 14 after adoptive transfer with 200 PFU LCMV-WE and killed on day 9 after LCMV infection for cytometry analysis. TCR transgenic cells are found in the TCR- $v_{\alpha 2}$ /CD8<sup>+</sup> compartment. The percentage of TCR- $v_{\alpha 2}$ -expressing CD8<sup>+</sup> cells is given in addition.

tions consisting mainly of CD8<sup>+</sup> T cells marked the sites of previous islets. In recipients of perforin-deficient spleen cells, in contrast, the islets had a hypertrophic aspect due to high numbers of mainly CD8<sup>+</sup>, but also CD4<sup>+</sup> infiltrating T lymphocytes (Fig. 6, bottom). Despite this very marked insulinitis, secretory cells, mostly scattered in groups of 5–10 cells surrounded by lymphocytic infiltrate, were still present. These cells reacted with an insulin-specific antibody demonstrating that they retained their capacity to produce insu-

lin and that the activated T cells migrating to the islets did not cause detectable destruction of  $\beta$  cells.

## Discussion

We have used a transgenic mouse model for autoimmune diabetes to evaluate the role of contact-dependent cytotoxicity vs. other effector mechanisms in  $\beta$  cell destruction and development of diabetes. In conclusion, our data indicate that perforin-dependent cytotoxicity is crucial to cause diabetes by eliminating islet  $\beta$  cells. Apparently other mechanisms such as  $\beta$  cell cytotoxic cytokines or Fas-dependent cytotoxicity are not efficient enough to fully compensate for the lack of perforin-dependent cytotoxicity. The result of the adoptive transfer experiment also indicates that perforin-dependent cytotoxicity is required in the late effector phase of the disease process and not in the early induction phase to start the inflammatory infiltration.

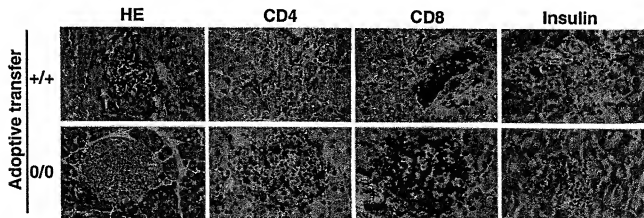
It was shown that the lack of functional perforin did not affect negative selection of  $V_{H6}$  expressing autoreactive T cells in Mls-1<sup>a</sup> perforin-deficient mice. Also, perforin-deficient LCMV-GP transgenic mice develop a similar LCMV-GP specific T cell response as nontransgenic perforin-deficient mice. Thus, together with the earlier observation that perforin-deficient mice have normal numbers of CD4, CD8, and NK marker positive cells (16), this indicated that the lack of functional perforin does not affect the development and the clonal deletion of T cells. This excluded any indirect effect of the gene disruption on the T cell repertoire and allowed the study of perforin-dependent cytotoxicity at the level of effector mechanisms.

We used two different protocols causing diabetes in the presence of perforin-expressing T cells. First, perforin-expressing and perforin-deficient LCMV-GP transgenic mice were infected with LCMV. This protocol leads to diabetes between day 9 and 11 in perforin-competent mice but failed to induce hyperglycemia in perforin-deficient

mice. In the second system, splenic T cells from either perforin-expressing or perforin-deficient mice were activated by an infection with vacc-LCMV-GP and subsequently adoptively transferred to LCMV-GP transgenic recipients. Again, the perforin-expressing cells induced readily diabetes whereas perforin-deficient spleen cells did not.

In the first experiment involving LCMV infection much fewer CD4<sup>+</sup> and CD8<sup>+</sup> T cells infiltrated the islets of perforin-deficient than normal control mice. This may be interpreted as evidence indicating that perforin-dependent cytotoxicity mediated by CD8<sup>+</sup> T cells leads to  $\beta$  cell damage and release of  $\beta$  cell-associated antigens, which in turn are taken up by local antigen presenting cells and activate CD4<sup>+</sup> T cells causing an inflammatory reaction eventually attracting additional cell populations (40). However, we think that the failure of LCMV infected perforin-deficient transgenic mice to develop marked infiltrations is rather explained by the failure of perforin-deficient mice to control the growth of LCMV (16, 18). This results in the persistence of high LCMV titers in many tissues after infection potentially diverting LCMV-specific effector cells and preventing them from infiltrating the LCMV-GP-expressing pancreatic islets.

In fact, this view is also strongly supported by the histological analysis of pancreatic sections following the adoptive transfer of activated LCMV-GP-specific T cells. Here, the antigenic load in normal control and perforin-deficient mice was similar due to the use of LCMV-GP recombinant vaccinia virus. A very marked infiltration of the islet consisting mainly of CD8<sup>+</sup> but also CD4<sup>+</sup> T cells developed in recipients of perforin-deficient spleen cells. This indicated that insulitis does not depend on the release of  $\beta$  cell proteins by perforin-dependent cytotoxicity. Recently, it was found that  $\beta_2$ -microglobulin-deficient NOD mice fail to develop diabetes and insulitis indicating that in the NOD model system, CD8<sup>+</sup> T cells are required for the initiation of the autoimmune islet-reactive process. Together with



**Figure 6.** Histological analysis of pancreatic islets from LCMV-GP transgenic mice after adoptive transfer with perforin-competent (+/+) or perforin-deficient (0/0) LCMV-GP-specific T cells. Spleen cells from vacc-LCMV-GP infected TCR transgenic mice were adoptively transferred into LCMV-GP transgenic recipients. Histological sections from recipients on 10 d after adoptive transfer were either stained with HE, CD4-, CD8-, or insulin-specific antibodies as indicated. The immunohistochemical stainings are rendered in black.  $\times 400$ .



our observation that insulinitis developed in the absence of perforin-dependent cytotoxicity this renders it very likely that CD8<sup>+</sup> T cells are involved in the initiation of diabetes by mechanisms other than perforin-dependent cytotoxicity such as secretion of cytokines (as for example IFN- $\gamma$  and TNF- $\beta$ ).

For the effector phase, however, our data clearly indicate that perforin-dependent cytotoxicity is necessary for  $\beta$  cell destruction and development of diabetes. The activities of other potential molecular effector mechanisms are apparently not sufficient to cause diabetes. These are Fas-dependent cytotoxicity, which is induced by LCMV infection of perforin-deficient LCMV-GP transgenic mice (Fig. 2), but may not be effective due to a lack of Fas-expression of islet cells (41), secretion of cytokines (14, 42), especially of IL-1, which has been found to be specifically toxic to  $\beta$  cells in vitro, and cytotoxic effects of reactive oxygen derivatives. We can not rule out that such alternative mechanisms can cause a limited degree of damage to  $\beta$  cells. But our results show that they are not efficient enough to cause the loss of more than 90% of  $\beta$  cells which is necessary to result in diabetes. This is also supported by the histological aspect of recipient islets after adoptive transfer of perforin-deficient cells that showed a dramatic infiltration of CD8<sup>+</sup> T cells into the islets without noticeable damage to  $\beta$  cells in the very same islet. Furthermore, TNF-receptor 1-deficient LCMV-GP transgenic mice are equally sensitive to LCMV-induced diabetes as TNF receptor 1-expressing control mice (McKall-Faienza, K., and P.S. Ohashi, manuscript in preparation), confirming the minor role of secreted TNF in the elimination of islet  $\beta$  cells.

The failure of non-perforin mechanisms to mediate tissue damage in diabetes is in accord with earlier findings in two model systems for virus induced immunopathological hepatitis. In LCMV- (16) as well as hepatitis B virus-induced hepatitis in mice (43) inactivation of perforin-dependent cytotoxicity abolished liver cell damage completely. Thus, analogous to the above findings for diabetes, viral hepatitis is mainly mediated by perforin-dependent cytotoxicity without measurable pathogenic contribution of the Fas-dependent cytotoxicity pathway. This is remarkable, because hepatocytes express significant levels of Fas and are extremely sensitive to injected Fas-specific monoclonal antibodies (44).

Independent earlier evidence pointed to the importance of cytotoxicity in the development of diabetes in the LCMV-GP model. It was found that diabetes develops on day 9–11 after LCMV infection, shortly after the LCMV-specific cytotoxic activity peaks on day 8. In addition, the disease develops in CD4<sup>+</sup> T cell- but not in CD8<sup>+</sup> T cell-depleted mice (45), consistent with a crucial role of the cytotoxic and a non-essential role of the helper T cell subset. In the NOD model system, it has been shown that after adoptive transfer of spleen cells from diabetic to young nondiabetic

NOD mice, perforin-expression in CD8<sup>+</sup> T cells peaks on day 12 preceding hyperglycemia by 1–2 d (46).

Which cells are responsible for the islet destruction by perforin-dependent cytotoxicity? Both CD8<sup>+</sup> T cells and NK cells are capable of mediating perforin-dependent cytotoxicity (16, 20), whereas CD4<sup>+</sup> T cells seem to exert cytotoxicity in vitro predominantly via the Fas-dependent pathway (47). The following evidence favors the CD8<sup>+</sup> T cells as being responsible for destruction of  $\beta$  cells by perforin-dependent cytotoxicity. First, in the LCMV-GP transgenic model, diabetes fails to develop after in vivo depletion of CD8<sup>+</sup> T cells (45). Second, only very few NK cells are found in the islet infiltrates in islets from diabetic NOD mice (1, 46) and from a freshly diabetic human (48). And finally, CD8<sup>+</sup> but not CD4<sup>+</sup> T cells, were found to express perforin in the islets of young diabetic NOD recipients after adoptive transfer of spleen cells from diabetic NOD mice (46).

These conclusions are somewhat contradictory to the findings that a CD4<sup>+</sup> T cell clone isolated from a diabetic NOD mouse induces diabetes in nondiabetic NOD mice (8) and that CD4<sup>+</sup> spleen cells from diabetic NOD mice transfer diabetes to NOD-*scid* mice (49). Immunohistological analysis of islets from diabetic NOD mice have shown that 60–70% of the infiltrating T cells were CD4<sup>+</sup> and only 30–40% CD8<sup>+</sup>. In human diabetes, in contrast, two independent reports have shown that the infiltrate is dominated by CD8<sup>+</sup> T cells with only very few CD4<sup>+</sup> T cells present. These analyses were performed by a first group with the pancreas of a 12-yr-old girl that died from ketoacidotic coma after having symptoms of diabetes for only a month (48) and by a second group investigating disease recurrence in three diabetic patients, who received pancreas grafts from their corresponding identical twins and in one patient whose graft was from a HLA identical sibling (50). In all of these cases, the islet infiltrating CD8<sup>+</sup> T cells outnumbered the CD4<sup>+</sup> T cells by far. Similarly, the insulinitis in LCMV-GP transgenic mice is dominated by CD8<sup>+</sup> T cells with ~70% CD8<sup>+</sup> vs 30% CD4<sup>+</sup> T cells. Thus, the islet infiltrate of LCMV-GP transgenic mice and of the described human cases have a similar prevalence of CD8<sup>+</sup> T cells, whereas the infiltrate in the islets of diabetic NOD mice contains more CD4<sup>+</sup> T cells. These findings raise the question whether CD4<sup>+</sup> T cells may play a more prominent role in the NOD system than in human diabetes.

In conclusion, we have shown here for a model system of autoimmune diabetes that insulinitis is not dependent upon tissue damage by perforin-dependent cytotoxicity, that perforin, which is expressed mainly by CD8<sup>+</sup> T cells in islet infiltrates, plays an important role in the destruction of islet  $\beta$  cells and that other mechanisms or soluble factors produced by T cells or macrophages fail to cause elimination of  $\beta$  cells with similar efficiency.

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